JOURNAL OF THE ROYAL SOCIETY Interface

Enzyme-sharing as a cause of multi-stationarity in signalling systems

Elisenda Feliu and Carsten Wiuf

J. R. Soc. Interface 2012 **9**, 1224-1232 first published online 2 November 2011
doi: 10.1098/rsif.2011.0664

JOURNAL OF THE ROYAL SOCIETY Interface

J. R. Soc. Interface (2012) **9**, 1224–1232
doi:10.1098/rsif.2011.0664
Published online 2 November 2011

Enzyme-sharing as a cause of multi-stationarity in signalling systems

Elisenda Feliu and Carsten Wiuf*

Bioinformatics Research Centre, Aarhus University, C. F. Møllers Allé 8, 8000 Aarhus, Denmark

Multi-stationarity in biological systems is a mechanism of cellular decision-making. In particular, signalling pathways regulated by protein phosphorylation display features that facilitate a variety of responses to different biological inputs. The features that lead to multi-stationarity are of particular interest to determine, as well as the stability, properties of the steady states. In this paper, we determine conditions for the emergence of multi-stationarity in small motifs without feedback that repeatedly occur in signalling pathways. We derive an explicit mathematical relationship φ between the concentration of a chemical species at steady state and a conserved quantity of the system such as the total amount of substrate available. We show that φ determines the number of steady states and provides a necessary condition for a steady state to be stable—that is, to be biologically attainable. Further, we identify characteristics of the motifs that lead to multi-stationarity, and extend the view that multi-stationarity in signalling pathways arises from multi-site phosphorylation. Our approach relies on mass-action kinetics, and the conclusions are drawn in full generality without resorting to simulations or random generation of parameters. The approach is extensible to other systems.

Keywords: steady state; kinase; stability; cross-talk; phosphorylation

1. INTRODUCTION

Multi-stationarity (the existence of more than one steady state under particular biological conditions) in cellular systems can be seen as a mechanism for cellular decision-making. How it arises is therefore fundamental to the understanding of cell signalling—that is, the communication of signals to regulate cellular activities and responses. Generally, cell signalling involves post-translational modifications of proteins, such as phosphorylation, acetylation or methylation. These modifications change the state of a protein in a discrete manner—for example, from an active to an inactive state.

In eukaryotes, reverse phosphorylation is the most frequent form of protein modification affecting approximately 30 per cent of all proteins in humans [1]. *Kinases* catalyse the transfer of phosphate groups to target proteins and *phosphatases* catalyse the reverse operation. After the completion of the human genome project, genome analysis estimated the number of kinases to approximately 500 [2], while the number of phosphatases is smaller by two-thirds [1]. Two protein phosphatases, PP-1 and PP-2A, account for the vast majority of all phosphatase activity [3] with more than 50 PP-1 targets being characterized [4].

As a consequence, there is a substantial complexity in the interplay between enzymes (kinases and phosphatases) and substrates, exemplified by systems where protein substrates use the same catalysing enzymes (enzyme-sharing) and systems where different enzymes catalyse the same reaction (enzyme competition). Competition and sharing are general examples of cross-talk between motifs.

The aim of this work is to determine the characteristics that lead to multi-stationarity. Following different modelling strategies, it has already been shown that feedback in signalling networks as well as multi-site phosphorylation can both account for multi-stationarity [5–7].

We present a mathematical approach for analysing the steady states of small systems. Our method leads to explicit conditions for when multi-stationarity occurs in terms of rate constants and conserved total amounts of substrates and enzymes. Further, the approach provides means to study the stability of steady states.

First, we present the motifs that we analyse and then we develop the method to determine multi-stationarity and to study stability. The paper concludes with some perspectives and discussion.

2. MOTIFS

2.1. Description

We analyse the motifs shown in figure 1. The motifs are referred to as Motif (a)–(l) and provide simple abstract representations of known cellular systems. Some examples motivating our choice of motifs are given in table 1. A rich source of examples is found in the well-studied mitogen-activated protein kinase (MAPK) cascades.

To understand how multi-stationarity relates to enzyme usage, we base our investigation on a motif that does not show multi-stationarity itself. Therefore, we

*Author for correspondence (wiuf@birc.au.dk).

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsif.2011.0664 or via http://rsif.royalsocietypublishing.org.

Received 29 September 2011
Accepted 12 October 2011

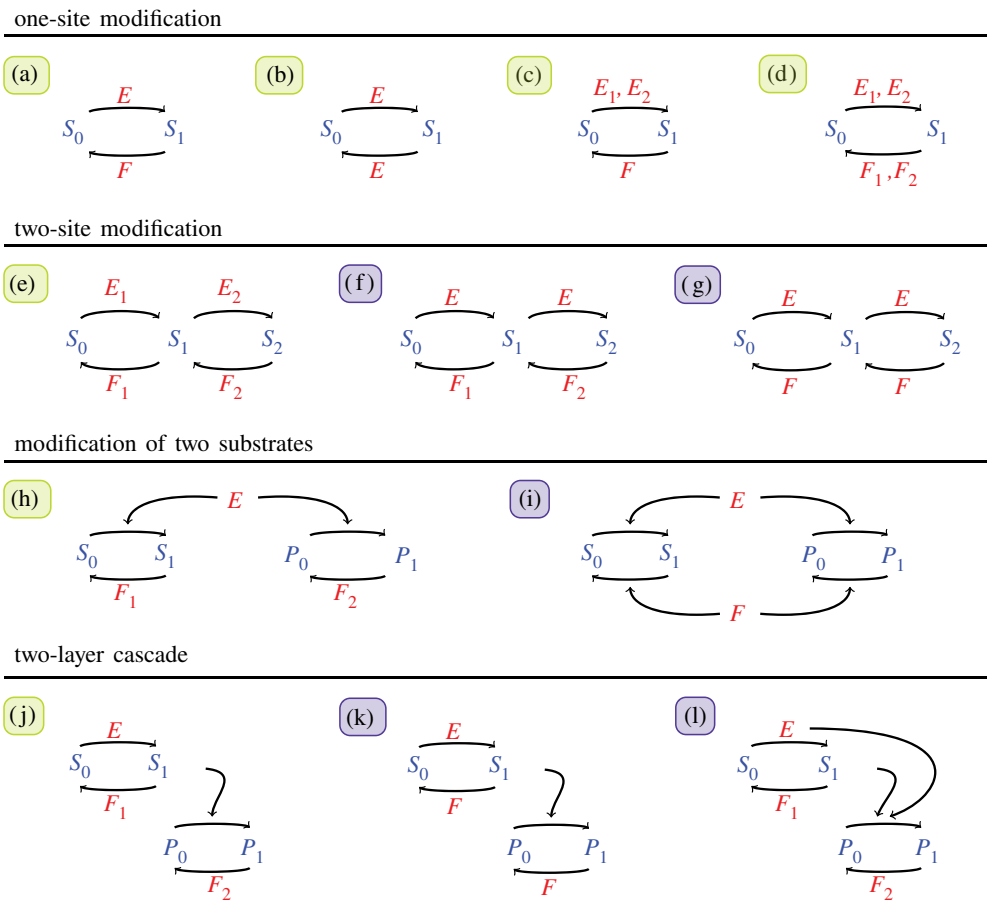

Figure 1. Motifs composed of one or two one-site cycles. Motifs with purple label, and only these, admit multiple biologically meaningful steady states. S_i and P_i are substrates with $i = 0,1,2$ phosphorylated sites. E, E_1, E_2 denote kinases, and F, F_1, F_2 phosphatases. In Motif (b), the kinase and the phosphatase are the same enzyme.

build the motifs from a one-site phosphorylation cycle which is monostable [16–19] and shown in Motif (a). A specific kinase (phosphatase) catalyses phosphorylation (dephosphorylation) and all modifications can be reversed. In general, protein phosphoforms are denoted by S and P (figure 1). If one phosphoform is converted into another, an arrow is drawn and the enzyme (E or F) catalysing the reaction is indicated.

Motifs (a)–(d) cover different possibilities for a one-site modification process. In Motif (b), the same enzyme catalyses phosphorylation and dephosphorylation. Motifs (c) and (d) account for competition between kinases and/or phosphatases to catalyse the same modification(s).

In eukaryotes, phosphorylation of most proteins takes place in more than one site [20], potentially with different biological effects [21]. Combination of two one-site cycles into a two-site sequential cycle yields three motifs: (e) all enzymes are different, (f) only one kinase but two phosphatases, and (g) one kinase and one phosphatase. By symmetry, Motif (f) represents as well a motif with one phosphatase but two kinases. We assume for simplicity that both phosphorylation and dephosphorylation proceed in a sequential and distributive manner [22]—that is, one site is (de)phosphorylated at a time in a specific order.

Motif (h) represents one-site modification of two substrates that share the same kinase but use different phosphatases. This motif represents by symmetry also a system with a shared phosphatase. If both the kinase and the phosphatase are shared, we obtain Motif (i).

Finally, two one-site modification cycles can be combined in a cascade motif, where the activated substrate of the first cycle acts as the kinase of the next. The interplay between enzymes is represented by three cascades: (j) dephosphorylation at each layer uses different phosphatases, (k) the phosphatase is not layer specific, and (l) the kinase of the first layer catalyses the modification in the second layer as well.

2.2. Mathematical modelling

We assume that any modification $S \to S^*$ follows the classical *Michaelis–Menten* mechanism in which an intermediate complex Z is formed reversibly but dissociates into product and enzyme G irreversibly:

$$S + G \underset{b}{\overset{a}{\rightleftarrows}} Z \xrightarrow{c} S^* + G$$

The phosphate donor, generally ATP, is assumed to be in large constant concentration and hence embedded into the rate constants. Imposing mass action kinetics, the species concentrations over time can be modelled by a system of polynomial differential equations. For example, in Motif (a) the equations are (here E also refers to the concentration of the kinase E, and similarly

Table 1. Cellular systems represented by Motifs (a)–(l).

Motifs	biological phenomena
(b)	a kinase acting also as phosphatase on the same substrate, e.g. HPrK/P kinase-phosphatase in Gram positive bacteria [8].
(c),(d)	several kinases and/or phosphatases acting on the same substrate, e.g. (i) several kinases phosphorylate the α subunit eukaryotic initiation factor (eIF2α) at Ser51 [9]; (ii) the phosphatases MAP kinase phosphatase 1 and protein tyrosine phosphatase STEP-like both modify Erk1 [10].
(e)	multi-site phosphorylation by different kinases and phosphatases at each site, e.g. (i) primed kinases, such as glycogen synthase kinase 3 [11]; (ii) Akt1 is (de)activated through three-site sequential (de)phosphorylation by three different kinases (phosphatases) [3].
(f),(g)	multi-site phosphorylation with the same kinase and/or phosphatase responsible for all modifications, e.g. (i) two-site phosphorylation of Erk catalysed by Mek; (ii) dephosphorylation of Erk2 catalysed by dual specific phosphatase 6 [12].
(h),(i)	the same enzyme catalysing the modification of two different substrates—e.g. the kinases Erk1, Erk2 and the kinase products of the p38 pathway catalyse phosphorylation of two substrates (the mitogen- and stress-activated protein kinase (MSK) 1/2 and the MAP kinase signal-integrating kinase (MNK) 1/2) [13].
(j),(k),(l)	cascades with several modification steps and substrates, e.g. (i) MAPK cascades; (ii) protein kinase A phosphorylates phosphorylase kinase, which in turn phosphorylates glycogen phosphorylase (with dephosphorylation carried out by the same phosphatase, PP-1, in the two different layers; [14, fig. 7.17] and [15]).

for the other species) as follows:

$$\dot{E} = (b^E + c^E)X - a^E E S_0 \quad \dot{X} = -(b^E + c^E)X + a^E E S_0$$

$$\dot{F} = (b^F + c^F)Y - a^F F S_1 \quad \dot{Y} = -(b^F + c^F)Y + a^F F S_1$$

$$S_0 = b^E X + c^F Y - a^E E S_0 \quad S_1 = c^E X + b^F Y - a^F F S_1,$$

where X (Y) is the intermediate complex formed by the enzyme E (F) and the substrate S_0 (S_1), and $\dot{x}$ denotes differentiation of $x = x(t)$ with respect to time. For all motifs, there are conservation laws that define time-conserved quantities (total amounts), e.g. $\dot{E} + \dot{X} = 0$ and so $\bar{E} = E + X$ is conserved. The total amounts are fixed by the initial concentrations and determine the state space of the dynamical system. Motif (a) has three conserved total amounts, namely $\bar{F} = F + Y$ and $\bar{S} = S_0 + S_1 + X + Y$ in addition to that of $\bar{E}$.

The steady states of the system are solutions (potentially with negative values) to the polynomial equations obtained by setting all derivatives to zero with the constraints imposed by the conservation laws, once total amounts have been fixed. These laws imply that some steady-state equations are redundant, e.g. either $\dot{E} = 0$ or $\dot{X} = 0$ can be disregarded. We focus on the *biologically meaningful steady states* (BMSSs), that is, the steady states for which all concentrations are non-negative (positive or zero). If at least two BMSSs exist for fixed total amounts, then the system is said to be multi-stationary.

The specific form of the chemical reactions for Motifs (a)–(l) together with the corresponding systems of differential equations are described in the electronic supplementary material.

3. THE STEADY-STATE FUNCTION φ

In this section, we outline the procedure used to analyse the motifs. Details of the mathematical analysis are in the electronic supplementary material.

The system of equations describing the steady states can be reduced substantially by elimination of variables [7,23]. For the motifs considered here, elimination of variables implies that the steady states are characterized by a relation $\bar{S} = \varphi(Y)$ between the concentration of one of the species, typically an intermediate complex Y, and the total amount of a substrate $\bar{S}$. The concentrations of the other species are given in terms of Y, usually as ratios of polynomials in Y. By imposing all concentrations to be non-negative, Y is restricted to a set Γ of possible values. Further, for any $\bar{S} \geq 0$, there is *at least one* BMSS, that is, $\bar{S} = \varphi(Y)$ for some Y in Γ. The function φ is continuous and differentiable in Γ and depends on the rate constants and the total amounts, except for $\bar{S}$.

The number of BMSSs can be found from the analysis of φ. If φ is strictly increasing or decreasing in Γ, φ is one-to-one and hence, for a given total amount $\bar{S}$, there is a corresponding unique Y at steady state. Consequently, multi-stationarity cannot occur (figure 2*a*).

Figure 2*b*,*c* shows situations where multi-stationarity occurs. If φ has increasing and decreasing parts, or if Γ is not connected, then $Y_1 \neq Y_2$ with $\varphi(Y_1) = \varphi(Y_2) = \bar{S}$ might exist. Hence, there are at least two BMSSs with the same $\bar{S}$.

These two figures represent substantially different switch responses. In figure 2*c*, there is only one BMSS for low $\bar{S}$. An increase of $\bar{S}$ to $\bar{S}_{\max}$ causes the system to switch to a 'high' steady state (high Y) under the assumption that the green steady states in the figure are stable. If $\bar{S}$ is decreased again to $\bar{S}_{\min}$, then the system switches back to a 'low' steady state. In figure 2*b*, there is one BMSS for low $\bar{S}$. An increase of $\bar{S}$ keeps the system in the first branch of φ and thus it will behave as a monostationary system.

Interestingly, the derivative $\varphi'(Y)$ of $\varphi(Y)$ provides means to determine whether some steady states are unstable. Unstable steady states are unattainable under biological conditions. Specifically, we find that either the regions in which φ is increasing or those in which it is decreasing must correspond to unstable steady states, see §5.

In summary, the function φ determines whether multiple BMSSs exist and encodes information about the stability of steady states. In §4, we analyse φ

for Motifs (e) and (f). We show how enzyme-sharing in a two-site cycle (f) leads to multi-stationarity, as opposed to a two-site cycle with different enzymes (e). A detailed analysis of all motifs is given in the electronic supplementary material.

4. MONO VERSUS MULTI-STATIONARITY

4.1. Monostationarity

Motifs (a)–(e), (h) and (j) have exactly one BMSS for any choice of rate constants and total amounts. In all cases, the function φ is increasing in Γ. The procedure is very similar in all cases and is thus only illustrated for Motif (e). We take some effort in explaining the details as the procedure might have general applicability.

Motif (e) consists of three phosphoforms of the substrate, S_0, S_1, S_2, with subscript indicating the number of phosphorylated sites. The chemical reactions of the system are:

$$S_0 + E_1 \underset{b_{1,E}}{\overset{a_{1,E}}{\rightleftarrows}} X_1 \xrightarrow{c_{1,E}} S_1 + E_1 \qquad S_1 + E_2 \underset{b_{2,E}}{\overset{a_{2,E}}{\rightleftarrows}} X_2 \xrightarrow{c_{2,E}} S_2 + E_2$$

$$S_1 + F_1 \underset{b_{1,F}}{\overset{a_{1,F}}{\rightleftarrows}} Y_1 \xrightarrow{c_{1,F}} S_0 + F_1 \qquad S_2 + F_2 \underset{b_{2,F}}{\overset{a_{2,F}}{\rightleftarrows}} Y_2 \xrightarrow{c_{2,F}} S_1 + F_2.$$

We denote the inverse of the Michaelis–Menten constants of E_i by $\kappa_{i,E} = a_{i,E}/(b_{i,E} + c_{i,E})$ and of F_i by $\kappa_{i,F} = a_{i,F}/(b_{i,F} + c_{i,F})$. The ratio of the catalytic constants of phosphatase and kinase is denoted by $\mu_i = c_{i,F}/c_{i,E}$.

The system has five conserved total amounts, which are assumed to be positive: four for the enzymes, $\bar{E}_i = E_i + X_i$ and $\bar{F}_i = F_i + Y_i$ $(i = 1,2)$, and one for the substrate, $\bar{S} = S_0 + S_1 + S_2 + X_1 + X_2 + Y_1 + Y_2$. The steady-state equations can be rewritten as

$$\left.\begin{array}{lll} X_1 = \kappa_{1,E} E_1 S_0 & Y_1 = \kappa_{1,F} F_1 S_1 & X_1 = \mu_1 Y_1 \\ X_2 = \kappa_{2,E} E_2 S_1 & Y_2 = \kappa_{2,F} F_2 S_2 & X_2 = \mu_2 Y_2. \end{array}\right\} \quad (4.1)$$

The last column gives X_i in terms of Y_i. The total amounts $\bar{E}_i, \bar{F}_i$ give E_i, F_i in terms of Y_i as well: $E_i = \bar{E}_i - \mu_i Y_i$, $F_i = \bar{F}_i - Y_i$. Further, if $\bar{E}_i, \bar{F}_i > 0$ then $E_i = 0$ or $F_i = 0$ cannot be solutions to equation (4.1). It follows that the concentrations E_i, F_i are positive if and only if Y_i is in $\Gamma_i = [0, \xi_i)$ with $\xi_i = \min(\bar{F}_i, \bar{E}_i/\mu_i)$.

We further isolate S_0, S_1 from the first row in equation (4.1) and S_2 from the second and obtain

$$S_0 = \frac{\mu_1 Y_1}{\kappa_{1,E}(\bar{E}_1 - \mu_1 Y_1)} \quad \text{and} \quad S_i = \frac{Y_i}{\kappa_{i,F}(\bar{F}_i - Y_i)} \quad (4.2)$$

for $i = 1,2$. Then, S_0, S_1 (respectively, S_2) are non-negative increasing continuous functions of Y_1 in Γ_1 (respectively, Y_2 in Γ_2). The remaining equation, $X_2 = \kappa_{2,E} E_2 S_1$, gives Y_2 in terms of Y_1:

$$Y_2 = f(Y_1) = \frac{\kappa_{2,E} \bar{E}_2 Y_1}{\mu_2(\kappa_{1,F}(\bar{F}_1 - Y_1) + \kappa_{2,E} Y_1)}. \quad (4.3)$$

The function f is non-negative increasing and continuous in Γ_1. Further, for Y_2 to be in Γ_2, it is required that Y_1 is in $\Gamma = [0, \xi) \subseteq \Gamma_1$ with $\xi = \min(\xi_1, f^{-1}(\xi_2))$.

Finally, using equation (4.3), we find that X_2 and S_2 are increasing functions of Y_1 in Γ. Therefore, using the earlier mentioned formulae, all concentrations at steady state are non-negative if and only if Y_1 is in Γ. We conclude that the BMSSs of the system satisfy

$$\bar{S} = S_0 + S_1 + S_2 + X_1 + X_2 + Y_1 + Y_2 = \varphi(Y_1)$$

for Y_1 in Γ. As φ is a sum of increasing continuous functions in Y_1, then so is φ. Additionally, $\varphi(0) = 0$ and $\varphi(Y_1)$ tends to infinity as Y_1 tends to ξ. Thus, φ has the form in figure 2*a* with a unique Y_1 for any given $\bar{S}$, that is, there is one BMSS.

4.2. Multi-stationarity

We consider a two-site phosphorylation system with one kinase but different phosphatases for each phosphoform, as shown in Motif (f). Multi-stationarity has been observed numerically in this system [6]. The system derives from Motif (e) by setting $E_1 = E_2$ and we use the notation introduced previously. The conservation laws are the same with the exception that there is only one kinase law, $\bar{E} = E + X_1 + X_2$. Define $\xi_i = \min(\bar{F}_i, \bar{E}/\mu_i)$ and $\Gamma_i = [0, \xi_i)$.

The system of equations to be solved is similar to equation (4.1) with $E = E_i$. Thus, we start by writing X_i, E, F_i as functions of Y_1, Y_2. Because E, F_i must be positive at any BMSS, we require $0 \leq Y_i < \bar{F}_i$ and $\mu_1 Y_1 + \mu_2 Y_2 < \bar{E}$. For these values we obtain

$$S_0 = \frac{\mu_1 Y_1}{\kappa_{1,E}(\bar{E} - \mu_1 Y_1 - \mu_2 Y_2)} \quad \text{and} \quad S_i = \frac{Y_i}{\kappa_{i,F}(\bar{F}_i - Y_i)}$$

for $i = 1,2$, which are non-negative increasing continuous functions of Y_i. Using $X_2 = \kappa_{2,E} E S_1$, we obtain Y_2 as a non-negative continuous function of Y_1 in Γ_1:

$$Y_2 = f(Y_1) = \frac{\kappa_{2,E}(\bar{E} - \mu_1 Y_1) Y_1}{\mu_2(\kappa_{1,F}(\bar{F}_1 - Y_1) + \kappa_{2,E} Y_1)}.$$

This function resembles that in equation (4.3) except for the quadratic term in the numerator, which is a consequence of the conservation law for $\bar{E}$ involving both Y_1 and Y_2. Further, f might not be increasing for all Y_1.

Let $\Gamma = \{Y_1 \in \Gamma_1, \text{ such that } f(Y_1) \in \Gamma_2\}$. Using the formulae derived earlier, all concentrations at steady state are non-negative if and only if Y_1 is in Γ. Hence, for any BMSS,

$$\bar{S} = S_0 + S_1 + S_2 + X_1 + X_2 + Y_1 + Y_2 = \varphi(Y_1)$$

with Y_1 in Γ. The function φ is continuous with $\varphi(0) = 0$ but Γ might not be a connected interval.

Define $\Lambda = (1 + \kappa_{2,E}/\kappa_{1,F})\mu_1 \bar{F}_1 - \bar{E}$. If $\Lambda \leq 0$, then f is an increasing function in Γ and we conclude that there is exactly one BMSS. If $\Lambda > 0$, then f has a unique local maximum for some α in Γ_1 and all cases in figure 2 can occur. By varying the value of $\bar{F}_2$ while keeping the other constants fixed, we obtain (figure 3):

— $\bar{F}_2 \leq (\bar{E} - \mu_1 \bar{F}_1)/\mu_2$ (orange): $\Gamma = [0, \alpha_1)$ with $f(\alpha_1) = \bar{F}_2$. The function f, and thus φ, are increasing and there is one BMSS (figure 2*a*).

— $(\bar{E} - \mu_1 \bar{F}_1)/\mu_2 < \bar{F}_2 \leq f(\alpha)$ (green): $\Gamma = [0, \alpha_1) \cup (\alpha_2, \xi_1)$ with $\alpha_1 \leq \alpha \leq \alpha_2$ and $f(\alpha_1) = f(\alpha_2) = \bar{F}_2$.

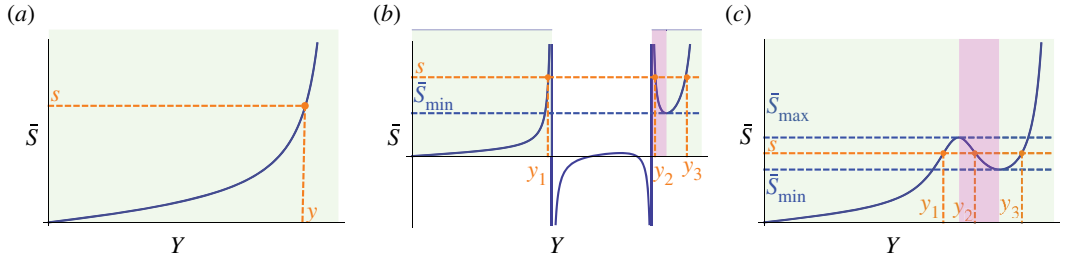

Figure 2. Possible shapes of φ in Γ (coloured regions: magenta = unstable BMSSs; green = (possible) stable BMSSs). (*a*) φ is increasing and for any s, there is one BMSS (y) such that $\varphi(y) = s$. (*b*) Γ consists of two disconnected regions. For $s < \bar{S}_{\min}$, there is one BMSS; for $s = \bar{S}_{\min}$ there are precisely two; and for $s > \bar{S}_{\min}$ there are three; φ is also defined in the white region but some concentrations become negative. (*c*) φ is in part decreasing, in part increasing. For $\bar{S}_{\min} < s < \bar{S}_{\max}$, there are three BMSSs; for $s = \bar{S}_{\min}$ or $s = \bar{S}_{\max}$, there are two; and for $s < \bar{S}_{\min}$ or $s > \bar{S}_{\max}$, there is one.

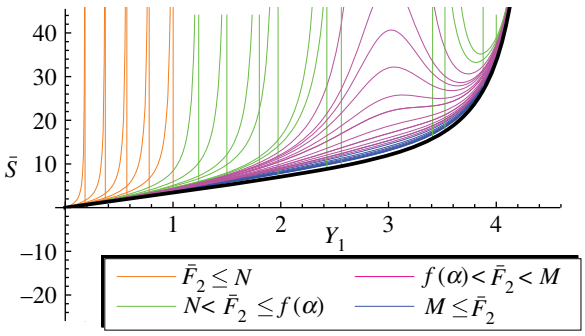

Figure 3. The function φ for Motif (f) for different values of $\bar{F}_2$ and fixed $\bar{E} > \mu_1 \bar{F}_1$ and $\Lambda > 0$. Let $N = (\bar{E} - \mu_1 \bar{F}_1)/\mu_2$. The values M and α depend on $\bar{E}$, $\bar{F}_1$ and the rate constants (see text). The set Γ is disconnected only when $N < \bar{F}_2 \leq f(\alpha)$. For large $\bar{F}_2$, φ approaches the black line. The vertical bars mark the boundary of Γ.

Hence, f is increasing in $[0,\alpha_1)$, decreasing in (α_2, ξ_1) and multi-stationarity occurs (figure 2*b*).

When $f(\alpha) < \bar{F}_2$, there is an M such that:

— $f(\alpha) < \bar{F}_2 < M$ (purple): $\Gamma = [0,\xi_1)$. The function φ has a decreasing part and multi-stationarity occurs (figure 2*c*).

— $M \leq \bar{F}_2$ (blue): $\Gamma = [0,\xi_1)$. The function φ is increasing and there is one BMSS (figure 2*a*).

4.3. Understanding multi-stationarity

Motifs (f), (g), (i), (k) and (l) exhibit multi-stationarity for some choices of total amounts and rate constants (figure 4). The regions for which multi-stationarity occurs are detailed in the electronic supplementary material. In Motifs (i), (k) and (l), multi-stationarity appears only as in figure 2*c*, while in Motifs (f) and (g) both forms in figure 2*b*, *c* occur.

It is remarkable that in Motifs (f), (k) and (l), multi-stationarity occurs for any set of rate constants and depends only on the initial conditions (that is, the total amounts). Thus, multi-stationarity can occur in these systems independently of the specific kinetics. In contrast, multi-stationarity in Motif (i) depends on the rate constants and hence not all kinetics exhibit multi-stationarity. The same appears to be the case for Motif (g) [24,25].

The common characteristic of these motifs is that a single enzyme is responsible for catalysing two different substrate modifications, which at the same time are *linked* (figure 4). Indeed, in Motifs (f) and (g), the substrates are linked through S_1, which is a modified as well as an unmodified substrate for the shared enzyme E. For the Motifs (k) and (l), the link is given by S_1, which is a modified substrate and a kinase, and the common enzymes are F and E, respectively. In Motif (i), the kinase E is common and the phosphatase F provides the link (or vice versa). In contrast, in Motif (h) an enzyme is responsible for two different modifications, but there is no link between the two substrates. Consequently, multi-stationarity cannot be observed.

Multi-stationarity can arise from two opposing dynamics acting on the same substrate (figure 4). For example, in Motif (f), if $\bar{F}_1$ is much bigger than $\bar{E}$ and $\bar{F}_2 < M$, then there are multiple BMSSs. Thus, because the amount of phosphatase in the first cycle is much larger than the amount of kinase, the substrate is pushed towards the unmodified form S_0, while in the second cycle, the substrate is driven towards the fully modified form S_2 (because $\bar{F}_2 < M$).

In Motif (i), provided the conditions on the parameters are fulfilled (figure 4), multi-stationarity occurs if either $\mu_1\bar{F} > \bar{E} > \mu_2\bar{F}$ or $\mu_2\bar{F} < \bar{E} < \mu_1\bar{F}$. It implies that in one cycle the phosphatase 'wins', while in the other the kinase does.

5. STABILITY ANALYSIS

BMSSs are defined as steady states for which all concentrations are non-negative. However, a steady state is biologically attainable only if it is (asymptotically) stable—that is, nearby trajectories are attracted to it. We show here for our motifs that if $\varphi'(Y) < 0$ for some steady-state $\varphi(Y) = \bar{S}$, then it is unstable.

5.1. The Jacobian and variable elimination

For a system of ordinary differential equations in $\mathbb{R}^m$, a steady-state z is asymptotically stable if all eigenvalues of the Jacobian evaluated at z have negative real parts [26, theorem 1.1.1]. Because the Jacobian is a real matrix, the complex eigenvalues come in pairs of conjugates and their product is a positive number. If m is odd and all eigenvalues have negative real parts, their product, and hence the determinant of the Jacobian, must be negative. If m is even and z stable, then the product of the eigenvalues must be positive. Thus, the sign

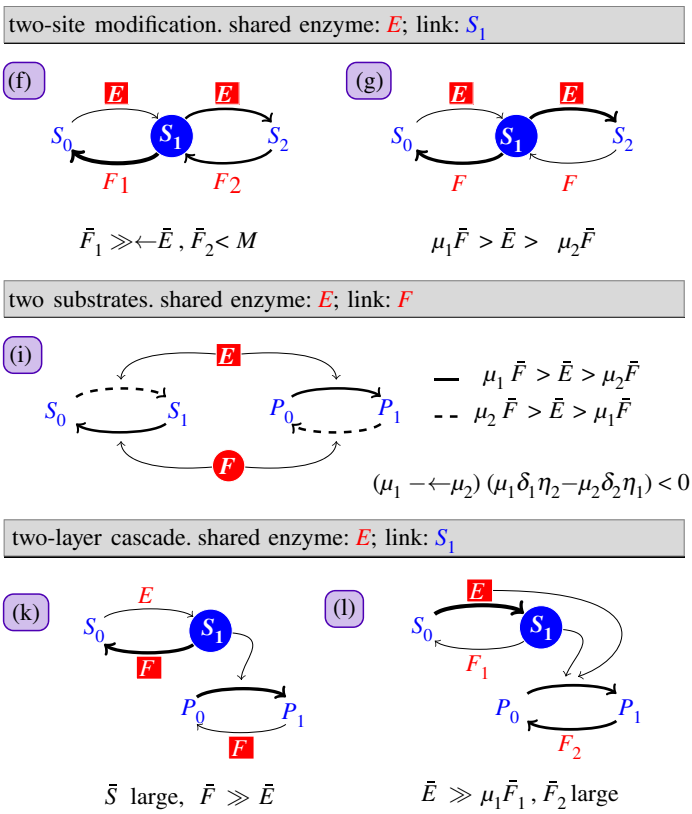

Figure 4. Conditions for multi-stationarity are given. The shared enzyme is marked with a coloured square; the link is marked with a coloured circle; predominant modifications are marked in bold. The symbol ≫ is short for 'much larger'.

of the determinant of the Jacobian provides a necessary condition for a steady state to be stable and a sufficient condition for it to be unstable.

For $x = (x_1, \ldots, x_n)$, let $x^{(j)} = (x_1, \ldots, \hat{x}_j, \ldots, x_n)$ (x with x_j removed). We make the following observation (see the electronic supplementary material for a proof): Let $f = (f_1, \ldots, f_n)$: $\Omega \subseteq \mathbb{R}^n \to \mathbb{R}^n$ be a differentiable function defined on an open set Ω and z such that $f(z) = 0$. Assume that x_j can be eliminated from the equation $f_i = 0$ in a neighbourhood around z; that is, there exists a differentiable function ψ: $\Omega^{(j)} \subseteq \mathbb{R}^{n-1} \to \mathbb{R}$, $z^{(j)}$ in $\Omega^{(j)} = \{x^{(j)} | x \in \Omega\}$, such that $x_j = \psi(x^{(j)})$ if $f_i(x) = 0$. Define $\bar{f}$: $\Omega^{(j)} \to \mathbb{R}^{n-1}$ by $\bar{f}_k(x) = f_k(x_1, \ldots, x_{j-1}, \psi(x), x_j, \ldots, x_{n-1})$ for all $k \neq i$ and let $\bar{J}$ denotes the associated Jacobian. Then, the determinant of the Jacobian of f at z satisfies

$$(-1)^{i+j} \frac{\partial f_i}{\partial x_j}(z) \quad \det(\bar{J}(z^{(j)})) = \det(J(z)). \qquad (5.1)$$

5.2. Unstable steady states

The relation between the sign of the determinant of the Jacobian and stability, together with equation (5.1), leads to a criterion to detect unstable steady states. For each motif, let $x = (x_1, \ldots, x_n)$ be the species concentrations, $\dot{x}_i = h_i(x)$ the differential equations and $\bar{A}_1 = g_1(x), \ldots, \bar{A}_c = g_c(x)$ the equations for the total amounts. We choose the order of the species such that x_i, $i = 1, \ldots, c$, can be isolated from $\bar{A}_i = g_i(x)$ and the steady-state equation $\dot{x}_i = 0$ becomes redundant. For fixed total amounts, $\bar{A}_1, \ldots, \bar{A}_c$, the steady states are the solutions to the system $f(x) = 0$ of n equations in n variables with $f_i(x) = g_i(x) - \bar{A}_i$ for $i = 1, \ldots, c$ and $f_i(x) = h_i(x)$ for $i = c+1, \ldots, n$.

Let $J(z)$ denote the Jacobian of f at z. In the electronic supplementary material we prove: if z is a steady state, that is, $f(z) = 0$, and either (i) $n - c$ is even and $\det(J(z)) < 0$ or (ii) $n - c$ is odd and $\det(J(z)) > 0$, then z is unstable. The proof relies on the observation made about the eigenvalues and equation (5.1).

The function φ of our motifs is derived through successive elimination of variables precisely from the system of equations $f(x) = 0$. Using equation (5.1), the sign of $\det(J(z))$ at a steady-state z can be traced back from the sign of the derivative of φ (the Jacobian of a system with one equation) by considering the equation number (i), the equation variable (j) and the sign of $\partial f_i / \partial x_j$ after each elimination.

To exemplify the procedure, consider Motif (f), where $n = 10$ and $c = 4$. The system is (see the electronic supplementary material for details):

$$\begin{aligned}
f_1(x) &= E + X_1 + X_2 - \bar{E} & f_6(x) &= X_2 - \kappa_{2,E} E S_1 \\
f_2(x) &= F_1 + Y_1 - \bar{F}_1 & f_7(x) &= X_1 - \mu_1 Y_1 \\
f_3(x) &= F_2 + Y_2 - \bar{F}_2 & f_8(x) &= X_2 - \mu_2 Y_2 \\
f_4(x) &= S_0 + S_1 + S_2 + X_1 & f_9(x) &= Y_2 - \kappa_{2,F} F_2 S_2 \\
&\quad + X_2 + Y_1 + Y_2 - \bar{S} & & \\
f_5(x) &= X_1 - \kappa_{1,E} E S_0 & f_{10}(x) &= Y_1 - \kappa_{1,F} F_1 S_1
\end{aligned}$$

with species $x = (E, F_1, F_2, S_0, X_1, X_2, S_1, S_2, Y_2, Y_1)$. The function φ is f_4 in terms of Y_2 after successive

Table 2. Elimination of variables for Motif (f). After each elimination the system $\bar{f}$ is rewritten to correctly determine the sign of $\partial \bar{f}_i / \partial x_j$ before the next elimination.

k	elimination	behaviour[a]	ϵ_k^b	k	elimination	behaviour	ϵ_k
1	(f_1, E)	$(1, 1, +)$	$+$	6	(f_5, S_0)	$(2, 1, -)$	$+$
2	(f_2, F_1)	$(1, 1, +)$	$+$	7	(f_6, S_1)	$(2, 1, -)$	$+$
3	(f_3, F_2)	$(1, 1, +)$	$+$	8	(f_9, S_2)	$(2, 1, -)$	$+$
4	(f_7, X_1)	$(4, 2, +)$	$+$	9	(f_{10}, Y_1)	$(2, 1, -)$	$+$
5	(f_8, X_2)	$(4, 2, +)$	$+$				

[a](i, j, σ) indicates that i, j are the indices of the equation and the variable iteratively being eliminated and σ shows whether $\bar{f}_i$ (f_i after substitution of the previous eliminations) is increasing ($\sigma = +$) or decreasing ($\sigma = -$) as a function of x_j.
[b]obtained as $\sigma(-1)^{i+j}$.

eliminations. Let $\epsilon_k = \pm 1$ depending on whether the sign of the determinant of the Jacobian changes $(-)$ or not $(+)$ after the k-th elimination. Then, $\mathrm{sign}(\varphi'(Y_2)) = (\prod_k \epsilon_k)\mathrm{sign}(\det(J(z)))$, where z is the steady state with $z_9 = Y_2$.

The order and sign of the eliminations are shown in table 2. We find that $\prod_k \epsilon_k = 1$, implying that the sign of $\varphi'(Y_2)$ agrees with the sign of the determinant of the Jacobian of f evaluated at the corresponding steady state. Because $n - c = 6$ is even, we conclude that the values of Y_2 for which φ is decreasing, that is, $\varphi'(Y_2) < 0$, correspond to unstable steady states. Further, it follows that unstable points come between other steady states that presumably are stable.

5.3. Stability in monostationarity motifs

The Routh–Hurwitz criterion [27] gives sufficient and necessary conditions for the Jacobian to have all eigenvalues with negative real parts. Thus, the (asymptotic) stability of a steady state can be determined by this criterion. For the Motifs (a)–(e) and (h), the criterion is fulfilled and the unique BMSS is asymptotically stable. We have not been able to determine whether the criterion is fulfilled for Motif (j).

6. DISCUSSION

We have investigated small motifs without feedback that account for cross-talk, enzyme competition, sharing and specificity in post-translational modification systems and determined some features that lead to multi-stationarity in signalling pathways.

Bistability, and generally multi-stability, in biological systems is seen as a mechanism of cellular decision-making. Compared with systems with a single steady state, the presence of multiple stable steady states provides a possible switch between different responses and increased robustness with respect to environmental noise. Our study has been driven by the observation that biological systems deviate from a one-to-one correspondence between enzymes and the modifications they catalyse. This phenomenon, known as cross-talk and enzyme-sharing, can cause multi-stationarity and hence be essential for regulating signalling systems.

Our work extends the view of multi-stationarity as arising from multi-site phosphorylation [7] to the view that multi-stationarity is driven by a single enzyme that catalyses linked substrates. Two opposing dynamics acting on the same substrate is a recurrent characteristic of multi-stationarity. These observations await a precise mathematical formulation and an investigation of its generality.

Our approach is conceptually simple and reduces to the study of analytical properties of a function φ that relates a conserved total amount and the concentration of a species at steady state. The graph $(\varphi(Y), Y)$ can be seen as a bifurcation diagram with one parameter, $\bar{S}$. When mono-stationarity occurs, analysis of φ is quite straightforward, while a more in-depth analysis is required when multi-stationarity occurs. An advantage of this approach is that unstable steady states are readily detected from the form of φ.

The existence of φ is not guaranteed in general. For instance, the function φ does not seem to exist for a three site modification cycle, that is, a motif resembling Motif (f) and Motif (g), but with an extra cycle allowing substrate S_2 to be modified to a substrate S_3. The existence of φ appears to be related to the number of reactions among the species rather than the number of species of the system. For instance, we have shown that for cascades of arbitrary length (extentions of Motif (j)) [23], as well as for arbitrary phosphorelays (not closely related to any of the motifs here) [28], the function φ exists.

In this work, the existence and computation of φ, and the determination of its domain, are obtained by direct manual inspection of each motif. Because each motif has its own particularities, it is unclear to us whether there is an automated procedure to determine the existence of φ and subsequently to compute it. For some of the motifs, φ could be chosen to relate another total amount or depend on another variable, while for other motifs only one variable seems to do the trick. For some motifs the function φ is rational and an explicit analytical description is available, while for other motifs it is not rational and its existence is derived from the Implicit Function Theorem. For some of the motifs, and after appropriately selecting the variable of φ, the command *Solve* in Mathematica provides rational functions expressing all other variables in terms of the selected one. In this case, however, it is still required to determine the domain of φ that ensures non-negativity of all concentrations at steady state by other means. We are currently investigating conditions that guarantee the existence of φ and algorithmic procedures to compute it.

Different mathematical procedures exist to rule out the existence of more than one BMSS. Two examples are found within the scope of chemical reaction network theory (CRNT) [29–31], which aims to understand dynamical properties of a system that depend on the network structure alone, and not on the specific reaction rates or total amounts. The first of them concerns the deficiency zero and one theorems [32,33] that provide conditions for the existence of a unique positive equilibrium and how it is approached for any set of total amounts. Motif (b) fulfils the assumptions of the deficiency zero theorem and we conclude that there exists a unique asymptotically stable positive steady state for any set of positive total amounts and fixed rate constants, and further that there is no non-trivial periodic orbit in the positive orthant. The other motifs do not satisfy the assumptions of the deficiency zero and one theorems.

The second approach concerns the so-called injective chemical networks taken with mass-action kinetics [34,35]. These were introduced by Craciun & Feinberg [34] for networks modelled in the context of a *continuous flow stirred tank reactor* (CFSTR) but the techniques apply more generally to networks that are not subject to conservation laws. In the study of Feliu & Wiuf [35], we extend the results to chemical reaction networks modelled with mass-action kinetics with conservation laws. An easy algorithmic criterion based on the Jacobian of the system of ODEs, similar to that in the study of Craciun & Feinberg [34], is provided. The motifs that do not allow multiple steady states are in fact injective in the sense of Feliu & Wiuf [35]. This implies that multi-stationarity cannot occur and that all steady states are non-degenerate. Using this approach, however, the existence of exactly one BMSS is not guaranteed, nor is the stability of the steady state. Further, if a network is not injective, then the existence of reaction rates and total amounts for which the system exhibits multi-stationarity cannot be concluded. Explicit values for which multi-stationarity occurs need to be found. The route taken in the present work provides a rationale to determine regions of multi-stationarity that is not based on random generation of parameter values. Additionally, information about the stability of the steady states is obtained as well. A remarkable difference between the present work and CRNT is the explicit use of the equations for the conservation laws and the specific values for the total amounts.

The results on injective networks have been extended to arbitrary kinetics fulfilling some mild properties [36,37] and dynamical systems modelling interacting species in general [38]. It turns out that our monostationarity motifs except Motif (b) fulfil the conditions for injectivity with arbitrary kinetics when considered as CFSTRs; that is, the stoichiometric matrix is strongly sign-determined [36]. In combination with the study of Craciun & Feinberg [39], we conclude that the motifs admit at most one (non-degenerate) positive steady state for any set of fixed total amounts. Some general considerations are provided about systems with feedback in Radde *et al.* [40].

Finally, within the theory of monotone dynamical systems [41], conditions can be given for a network to admit one globally asymptotic positive steady state for a given set of total amounts [42]. One of the assumptions is that no species take part in more than two reactions, which is only fulfilled by Motif (a).

The mathematical approach we have taken to analyse the motifs requires manual intervention. When this route can be pursued, the BMSSs are the solutions to a single equation $\bar{S} = \varphi(Y)$. Analysis of φ enables multi-stationarity to be determined even in situations where the number of parameters is large. Further, the steady-state concentrations of all species are given in terms of the steady state of the selected species (Y); in many cases, this relationship can be stated as a rational function. Further, the approach enables comprehensive studies of qualitative features of the system, such as the system's response to variations in total amounts or sensitivity in stimulus-response curves, independently of rate constants [23,28,43].

E.F. is supported by a post-doctoral grant from the 'Ministerio de Educación' of Spain and the project MTM2009-14163-C02-01 from the 'Ministerio de Ciencia e Innovación'. C.W. is supported by the Lundbeck Foundation (Denmark), the Carlsberg Foundation (Denmark), the Leverhulme Trust (UK) and the Danish Research Councils. Neil Bristow, Freddy Bugge Christiansen and Michael Knudsen are thanked for commenting on the manuscript.

REFERENCES

1 Cohen, P. 1989 The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58**, 453–508. (doi:10.1146/annurev.bi.58.070189.002321)
2 Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. 2002 The protein kinase complement of the human genome. *Science* **298**, 1912–1934. (doi:10.1126/science.1075762)
3 Xiao, L. *et al.* 2010 Protein phosphatase-1 regulates Akt1 signal transduction pathway to control gene expression, cell survival and differentiation. *Cell Death Differ.* **17**, 1448–1462. (doi:10.1038/cdd.2010.16)
4 Cohen, P. T. 2002 Protein phosphatase 1-targeted in many directions. *J. Cell Sci.* **115**, 241–56.
5 Kapuy, O., Barik, D., Domingo Sananes, M. R., Tyson, J. J. & Novák, B. 2009 Bistability by multiple phosphorylation of regulatory proteins. *Prog. Biophys. Mol. Biol.* **100**, 47–56. (doi:10.1016/j.pbiomolbio.2009.06.004)
6 Markevich, N. I., Hoek, J. B. & Kholodenko, B. N. 2004 Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. *J. Cell Biol.* **164**, 353–359. (doi:10.1083/jcb.200308060)
7 Thomson, M. & Gunawardena, J. 2009 Unlimited multistability in multisite phosphorylation systems. *Nature* **460**, 274–277. (doi:10.1038/nature08102)
8 Chaptal, V., Vincent, F., Gueguen-Chaignon, V., Monedero, V., Poncet, S., Deutscher, J., Nessler, S. & Morera, S. 2007 Structural analysis of the bacterial HPr kinase/phosphorylase V267F mutant gives insights into the allosteric regulation mechanism of this bifunctional enzyme. *J. Biol. Chem.* **282**, 34 952–34 957. (doi:10.1074/jbc.M705979200)
9 de Haro, C., Mendez, R. & Santoyo, J. 1996 The eIF-2α kinases and the control of protein synthesis. *FASEB J.* **10**, 1378–1387.
10 Shaul, Y. D. & Seger, R. 2007 The mek/erk cascade: from signaling specificity to diverse functions. *Biochim. Biophys. Acta* **1773**, 1213–26. (doi:10.1016/j.bbamcr.2006.10.005)

11 Cohen, P. 2000 The regulation of protein function by multisite phosphorylation—a 25 year update. *Trends Biochem. Sci.* **25**, 596–601. (doi:10.1016/S0968-0004(00)01712-6)

12 Ferrell, J. E. & Bhatt, R. R. 1997 Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. *J. Biol. Chem.* **272**, 19008–16. (doi:10.1074/jbc.272.30.19008)

13 Keshet, Y. & Seger, R. 2010 The map kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods Mol. Biol.* **661**, 3–38. (doi:10.1007/978-1-60761-795-2_1)

14 Fell, D. 1997 *Understanding the control of metabolism*. London, UK: Portland Press.

15 Cohen, P. 1992 Signal integration at the level of protein kinases, protein phosphatases and their substrates. *Trends Biochem. Sci.* **17**, 408–413. (doi:10.1016/0968-0004(92)90010-7)

16 Goldbeter, A. & Koshland, D. E. 1981 An amplified sensitivity arising from covalent modification in biological systems. *Proc. Natl Acad. Sci. USA* **78**, 6840–6844. (doi:10.1073/pnas.78.11.6840)

17 Goldbeter, A. & Koshland, D. E. 1984 Ultrasensitivity in biochemical systems controlled by covalent modification. Interplay between zero-order and multistep effects. *J. Biol. Chem.* **259**, 14 441–14 447.

18 Bluthgen, N., Bruggeman, F. J., Legewie, S., Herzel, H., Westerhoff, H. V. & Kholodenko, B. N. 2006 Effects of sequestration on signal transduction cascades. *FEBS J.* **273**, 895–906. (doi:10.1111/j.1742-4658.2006.05105.x)

19 Salazar, C. & Höfer, T. 2006 Kinetic models of phosphorylation cycles: a systematic approach using the rapid-equilibrium approximation for protein–protein interactions. *Biosystems* **83**, 195–206. (doi:10.1016/j.biosystems.2005.05.015)

20 Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P. & Mann, M. 2006 Global, *in vivo*, and site-specific phosphorylation dynamics in signaling networks. *Cell* **127**, 635–648. (doi:10.1016/j.cell.2006.09.026)

21 Wu, R. C., Qin, J., Yi, P., Wong, J., Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. 2004 Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic reponses to multiple cellular signaling pathways. *Mol. Cell* **15**, 937–949. (doi:10.1016/j.molcel.2004.08.019)

22 Salazar, C. & Höfer, T. 2009 Multisite protein phosphorylation—from molecular mechanisms to kinetic models. *FEBS J.* **276**, 3177–3198. (doi:10.1111/j.1742-4658.2009.07027.x)

23 Feliu, E., Knudsen, M., Andersen, L. N. & Wiuf, C. 2011 An algebraic approach to signaling cascades with n layers. *Bull. Math. Biol.* (doi:10.1007/s11538-011-9658-0).

24 Conradi, C., Flockerzi, D. & Raisch, J. 2008 Multistationarity in the activation of a MAPK: parametrizing the relevant region in parameter space. *Math. Biosci.* **211**, 105–131. (doi:10.1016/j.mbs.2007.10.004)

25 Wang, L. & Sontag, E. D. 2008 On the number of steady states in a multiple futile cycle. *J. Math. Biol.* **57**, 29–52. (doi:10.1007/s00285-007-0145-z)

26 Wiggins, S. 2003 Introduction to applied nonlinear dynamical systems and chaos, 2nd edn. New York, NY: Springer.

27 Hurwitz, A. 1996 Über die Bedingungen, unter welchen eine Gleichung nur Wurzeln mit negativen reellen Theilen besitzt. In *Stability theory* (*Ascona, 1995*). International Series of Numerical Mathematics, vol. 121, pp. 239–249. Basel: nBirkhäuser. (Reprinted by *Math. Ann.* (1895), 273–284 [JFM 26.0119.03]).

28 Knudsen, M., Feliu, E. & Wiuf, C. 2011 Exact analysis of intrinsic qualitative features of phosphorelays using mathematical models. (http://arxiv.org/abs/1109.5159)

29 Horn, F. & Jackson, R. 1972 General mass action kinetics. *Arch. Rational Mech. Anal.* **47**, 81–116. (doi:10.1007/BF00251225)

30 Feinberg, M. 1980 *Lectures on chemical reaction networks*. See http://www.che.eng.ohio-state.edu/~Feinberg/LecturesOnReactionNetworks.

31 Feinberg, M. & Horn, F. J. M. 1977 Chemical mechanism structure and the coincidence of the stoichiometric and kinetic subspaces. *Arch. Rational. Mech. Anal.* **66**, 83–97. (doi:10.1007/BF00250853)

32 Feinberg, M. 1987 Chemical reaction network structure and the stability of complex isothermal reactors. I. The deciency zero and deciency one theorems. *Chem. Eng. Sci.* **42**, 2229–2268. (doi:10.1016/0009-2509(87)80099-4)

33 Feinberg, M. 1995 The existence and uniqueness of steady states for a class of chemical reaction networks. *Arch. Rational Mech. Anal.* **132**, 311–370. (doi:10.1007/BF00375614)

34 Craciun, G. & Feinberg, M. 2005 Multiple equilibria in complex chemical reaction networks. I. The injectivity property. *SIAM J. Appl. Math.* **65**, 1526–1546. (doi:10.1137/S0036139904440278)

35 Feliu, E. & Wiuf, C. 2011 Injectivity of chemical reaction networks with mass action kinetics revisited. (http://arxiv.org/abs/1109.5149)

36 Banaji, M., Donnell, P. & Baigent, S. 2007 P matrix properties, injectivity, and stability in chemical reaction systems. *SIAM J. Appl. Math.* **67**, 1523–1547. (doi:10.1137/060673412)

37 Banaji, M. & Craciun, G. 2010 Graph-theoretic criteria for injectivity and unique equilibria in general chemical reaction systems. *Adv. Appl. Math.* **44**, 168–184. (doi:10.1016/j.aam.2009.07.003)

38 Banaji, M. & Craciun, G. 2009 Graph-theoretic approaches to injectivity and multiple equilibria in systems of interacting elements. *Commun. Math. Sci.* **7**, 867–900.

39 Craciun, G. & Feinberg, M. 2006 Multiple equilibria in complex chemical reaction networks: extensions to entrapped species models. *Syst. Biol.* (*Stevenage*) **153**, 179–186. (doi:10.1049/ip-syb:20050093)

40 Radde, N., Bar, N. S. & Banaji, M. 2009 Graphical methods for analysing feedback in biological networks: a survey. *Int. J. Syst. Sci.* **41**, 35. (doi:10.1080/00207720903151326)

41 Smith, H. L. 1995 *Monotone dynamical systems. Mathematical surveys and monographs*, vol. 41. Providence, RI: American Mathematical Society.

42 Angeli, D., De Leenheer, P. & Sontag, E. 2010 Graph-theoretic characterizations of monotonicity of chemical networks in reaction coordinates. *J. Math. Biol.* **61**, 581–616. (doi:10.1007/s00285-009-0309-0)

43 Feliu, E., Knudsen, M. & Wiuf, C. In press. Signaling cascades: consequences of varying substrate and phosphatase levels. In *Advances in systems biology*, vol. 736 (eds I. I. Goryanin & A. B. Goryachev). Berlin, Germany: Springer.